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C2C
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(71) Applicants
Anthony Charles Richardson
2 Norman Avenue, Henley-on-Thames, Oxon.
Percy Francis George Praill
16 Milton Road, Ickenham, Uxbridge, Middlesex
Robert Graham Price
11 Hillside Road, Northwood, Middlesex
(72) Inventors
Anthony Charles Richardson
Percy Francis George Praill
Robert Graham Price
(74) Agent
R. S. Crespi,

(54) **Enzyme assay reagents and apparatus for use in the assay of enzymes**

(57) An enzyme assay reagent comprises a substance which is capable of interaction with the enzyme to give rise to the formation of a compound of formula X-A-Y-NO_2, wherein A comprises an aromatic nucleus, X comprises an auxochromic group and Y comprises an unsaturated group which is capable of transmitting electron resonance between the aromatic nucleus and the nitro substituent, said compound *per se* being capable of producing a visual signal which is easily discernible by eye.

An apparatus for use in the assay of an enzyme is in the form of a through-flow device and comprises two interconnectable compartments, an incubation compartment containing reagent associated with solid phase material, and a visualisation compartment containing a solid phase reagent which presents an alkaline environment to the compound of formula X-A-Y-NO_2.

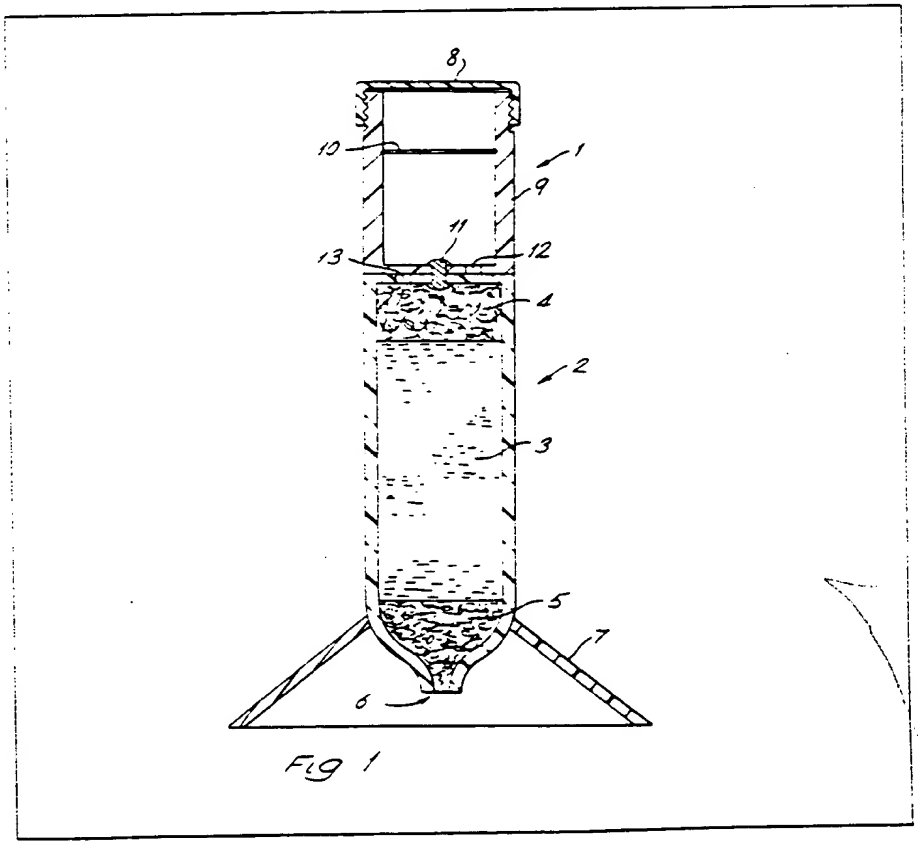

Fig 1

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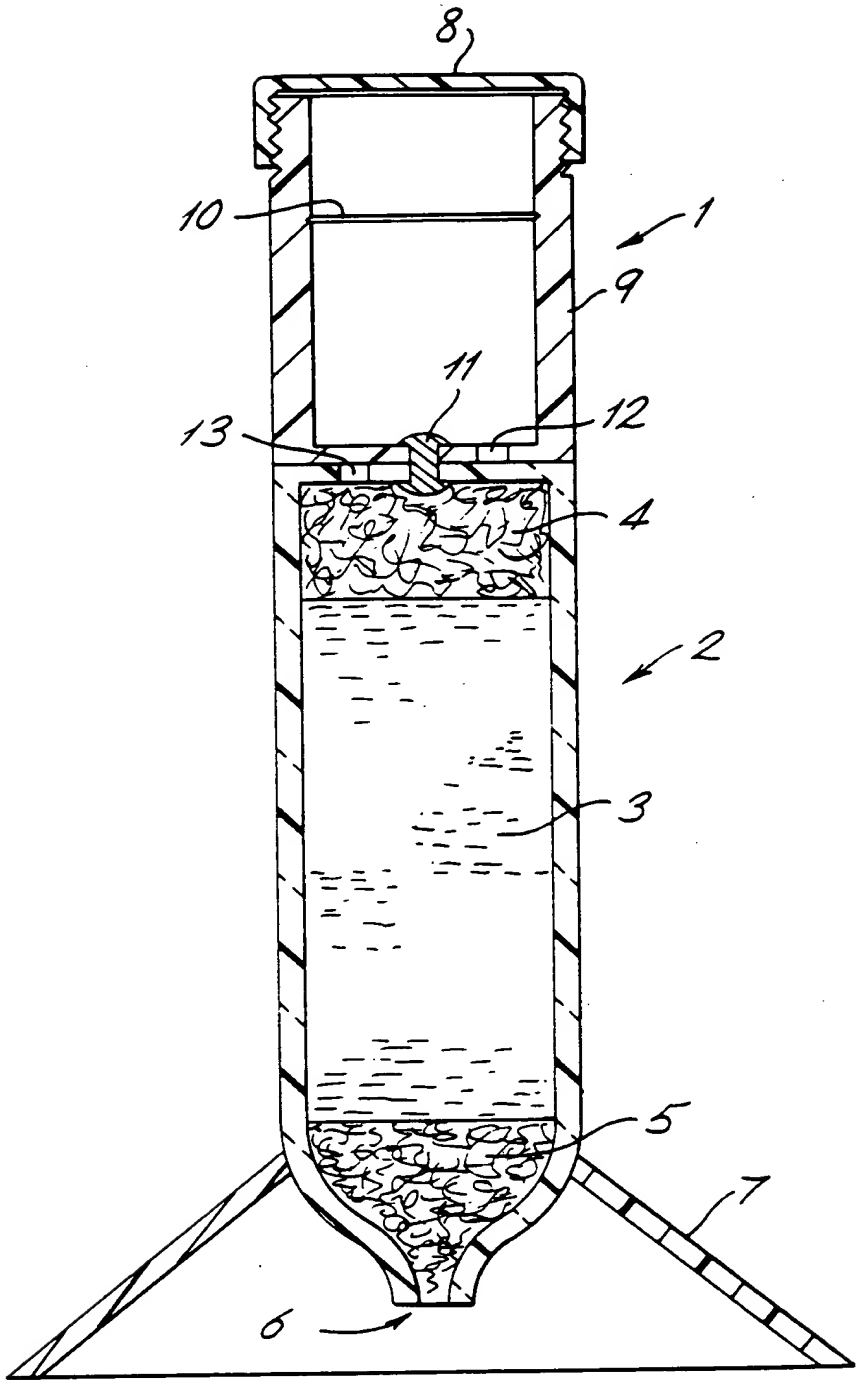


Fig. 1

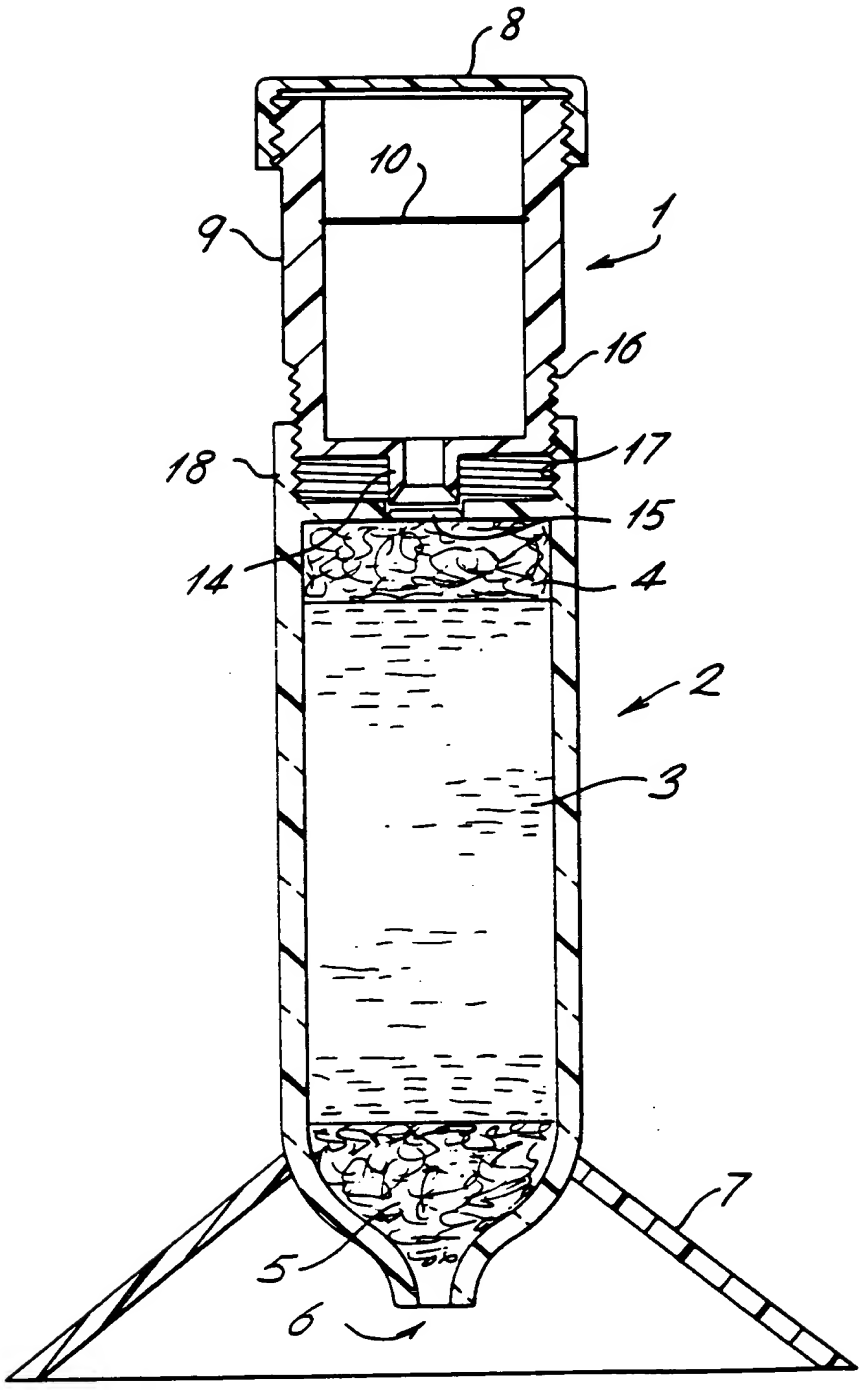


Fig. 2

SPECIFICATION

Enzyme assays

This invention relates to enzyme assays, and in particular to reagents for the detection and determination of enzymes.

The presence or absence of certain enzymes in phsiological samples, such as urine or serum samples from human patients, is a valuable indicator of illness or deficiencies in the organism concerned e.g. organ malfunctions in human patients. For example, increases in the level of the enzyme N-acetyl-β-D glucosaminidase (NAG) excreted in the urine of "kidney transplant" patients is an early sign of impending rejection of the transplanted kidney, besides being a general indicator of renal and other diseases. At present the customary clinical assay for NAG involves incubation of a sample of urine with the 4-methyl-umbelliferyl glycoside substrate of the enzyme which interacts to release the corresponding umbelliferone which is then monitored fluorimetrically. Alternatively, a nitrophenyl glycoside substrate, p-nitrophenyl-2- acetamido-2-deoxy- β-D-glucopyranoside, may be used, interacting with the enzyme to release p-nitrophenol which is monitored colorimetrically. Both these assays, however, require the use of sophisticated spectrophotometric apparatus and thus are only suitable for use when there is access to well-equipped laboratory facilities.

New enzyme assay reagents have now been devised which, in some cases, may be employed without requiring the use of sophisticated monitoring apparatus.

According to the present invention an enzyme assay reagent comprises a substance which is capable of interaction with the enzyme to give rise to the formation of a compound of formula X-A-Y-NO_2, wherein A comprises an aromatic nucleus, X comprises an auxochromic group and Y comprises an unsaturated group which is capable of transmitting electron resonance between the aromatic nucleus and the nitro substituent, said compound *per se* being capable of producing a visual signal which is easily discernible by eye.

The invention also includes a method for the assay of an enzyme which comprises incubating a sample containing the enzyme with a reagent according to the invention to give rise to the production of a compound of formula X-A-Y-NO_2 as hereinbefore defined, and, if necessary, subjecting the compound to further treatment to develop a visual signal which is easily discernible by eye.

The reagent typically comprises an enzyme substrate portion and a further portion which gives rise to the formation of the compound of formula X-A-Y. Usually the reagent of the invention comprises the compound X-A-Y-NO_2, or a simple precursor therefor, in combination with the substrate portion, and on interaction with the enzyme the substance releases the compound or simple precursor. Thus the compound or precursor is usually attached to the substrate portion at or adjacent to the side within the substance at which enzyme activity takes place. In one embodiment the enzyme substrate portion and further portion of the reagent are conveniently linked through the auxochromic group X and the substance has the general formula S-X-A-Y-NO_2, wherein S comprises the enzyme substrate portion of the substance and X, A and Y are as previously defined, and such reagents release the compound on interaction with the enzyme.

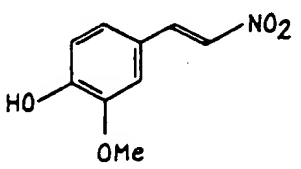
The reagents, substances and method of the present invention are widely applicable to the assay of enzymes in general, the enzyme substrate portion of the substance being varied in accordance with the particular enzyme which it is desired to assay. Typically, however, the enzymes which may be assayed by the invention are catabolic enzymes, such as those which play an important role in the breakdown of macromolecules in cellular tissues. For example, enzymes which may be assayed include carboxylate esterases and lipases, acid and alkaline phosphatases, phosphodiesterases and sulphateses, and thus corresponding reagents may comprise appropriate carboxylate, phosphate, diphosphate and sulphate esters. Also, in particular, the invention is applicable to the assay of glycosidases in which case the substrate portion S of the reagent characteristically comprises a corresponding glycoside carbohydrate substituent, such as an α or β-glucopyranosyl, -galactopyranosyl, or -mannopyranosyl substituent. For example, the invention has been found to be particularly suitable for application to the assay of N-acetyl-β-D-glucosaminidase (NAG).

The invention may be employed to assay for the presence of enzymes in general and to monitor their concentration levels; for instance, for diagnosis of a corresponding defect or illness. For example, the NAG content of urine may be assayed for early detection of kidney disease, or in the specific case as an early indicator of kidney rejection in "kidney transplant" patients. Alternatively, the invention may be used to determine the absence of enzymes which may, for instance, be indicative of certain genetic disorders. Generally, also, the reagents and method of the invention may provide convenient tools for use in biochemical and medical research.

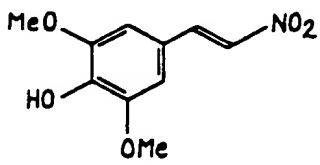
Preferably the compound X-A-Y-NO_2 is capable of producing a colour change which is easily discernible to the eye, and in this latter respect the colour produced is typically substantially different from background colouration of the sample. For example, reagents of the invention for use with urine samples, such as reagents for assay of NAG, typically release substances capable of producing colours substantially different from the background colour of urine. Preferably the reagents of the invention release compounds which are capable of producing distinctive colours, preferably reds or blues. Thus suitable re-

The existence of electron donating substituents, such as alkoxy groups e.g. methoxy, adjacent to the hydroxyl substituent appears to stabilise the formation of this radical ion, as is borne out by the colour properties of the 3-methoxy and especially the 3,5-dimethoxy analogues shown below as formulae III and IV respectively, upon which particularly preferred reagents are based.

III

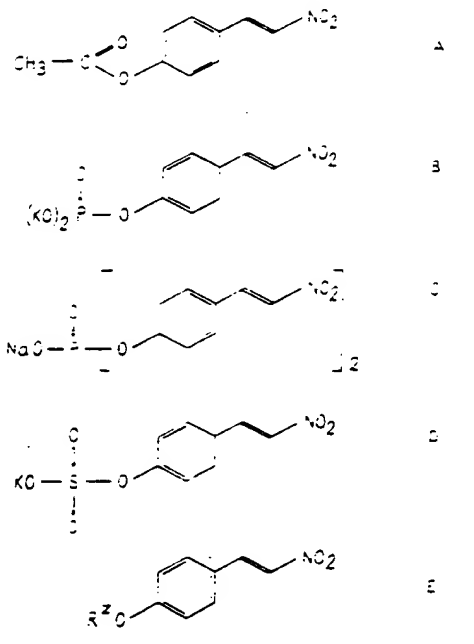


IV



Thus the mono-methoxy compound III and the di-methoxy compound IV have absorbance maxima (λ max) of 506 and 530 with extinction co-efficients (ε) of 24,&600 and 22,300 respectively in buffer at pH 8.8, and exhibit satisfactory scarlet-red colourations under these conditions.

Conveniently the auxochromic group X e.g. -OH, may provide the linkage between the compound X-A-Y-NO_2 and the substrate portion of the reagent, such that the compound is released on interaction with the enzyme. Thus examples of reagents according to the invention are given below by formulae A to E, referring, for the sake of simplicity, only to reagents comprising a simple 4-hydroxy-ω-nitrostyryl substituent. It will be appreciated, however, that this latter substituent may be replaced by a substituent relating to the compounds X-A-Y-NO_2 in general.



being reagents suitable for assay of (A) esterases or lipases, (B) acid or alkaline phosphatases, (C) phosphodiesterases, (D) arysulphatases and (E) glycosidases respectively. R^z in formula E denotes a carbohydrate substituent, such as an α or β glucopyranosyl, galactopyranosyl or mannopyranosyl.

In accordance with the invention, it has been found that the preferred nitrostyryl substituted reagents may be prepared from vanillin or similar hydroxy benzaldehyde based materials such as p-hydroxybenzaldehyde or salicyladehyde by nitromethylation of the corresponding substrate-vanillin or -similar compound adduct. Preferably mildly alkaline conditions are employed during nitromethylation. It is believed

for example, that use of 3% of the appropriate NAG detecting glycoside (E) on an inert cellulose solid phase in combination with DEAE cellulose visualisation is capable of detecting a threshold concentration of NAG in urine from about 40 – 50 n mol/ml after incubation at 37° for twenty minutes; whereas, use of cellulose containing 8% of the glycoside in the same system gives a threshold of detection of about 20 – 30 n mol/ml. Both systems give broad intense bands for abnormal urines which are easily distinguished from those given by normal urines. It will be appreciated, therefore, having regard to the effect of substrate concentration and the ease of visualisation, that the method of the invention may be conveniently performed by patients themselves in their homes using a suitably prepared through-flow device and requires minimal analytical skills. For example, the patient may simply add a fixed quantity of fresh urine to the incubation compartment e.g. up to a fixed level indicated on the side of the compartment, and after a standard incubation period, which will usually depend upon the ambient temperature, connect the incubation and visualisation compartments permitting incubated urine to flow through the solid phase visualising reagent.

The visual signal, e.g. colour change, produced by the released substance advantageously provides a simple and quick means of detecting the presence of the enzyme in the sample as compared with the methods previously used which rely upon sophisticated monitoring apparatus. Also the intensity of the visual signal e.g. colour, produced may conveniently be used as a rough visual indicator of the concentration of enzyme present in the sample. If more accurate measurements are required, however, the reagents of the invention may be monitored spectrophotometrically, for instance in the conventional manner. For example, the colour produced may be estimated using a colourimeter, usually after adjustment of pH to 8.5 - 9.5 with an appropriate buffer, and, in such methods, the present reagents may be more suitable than corresponding prior art reagents, such as p-nitrophenolic and *o*- nitrophenolic substrates.

The invention is further described by way of illustration only in the following examples and description which relate to the preparation of enzyme assay reagents according to the invention and components thereof and to their use in the method of the invention, the description also referring to the accompanying diagrams in which:–

Figure 1 represents one form of flow-through device according to the invention; and

Figure 2 represents an alternative form of flow-through device according to the invention.

Example I *Preparation of nitrostyryl glycosides for detection and determination of corresponding glycosidases*

I Preparation of hydroxy-benzaldehyde glycosides

As a first stage in the preparation of nitrostyryl glycoside enzyme substrates according to the invention the appropriate hydroxy-benzaldehyde glycosides are prepared.

A solution of the appropriate glycopyranosyl halide (70 mmole) in acetone (200 ml) was treated with a solution of the phenol (100 mmole) in *M*-sodium hydroxide, the resulting mixture being maintained at room temperature with stirring for a period of about 16 hours. The reaction mixture was then diluted with water until the product separated. Crystalline glycoside products were filtered off directly, and non-crystalline products were isolated by chloroform extraction in the usual manner.

After crystalline products have been filtered off, further product may be recovered from the mother liquors by extraction with chloroform or dichloromethane.

The results obtained during the preparation of a range of hydroxy-benzaldehyde glycosides are given below.

(a) 4-(2-*acetamido*- 3,4,6,*tri*-O-*acetyl*-2- *deoxy*-α-D-*gluco*- *pyranosyloxy*)-3- *methoxybenzaldehyde* was obtained in 55% yield from the 2-acetamido-3,4,6- tri-*O*-acetyl-2-deoxy- α-D-gluco-pyranosyl chloride and had m.p. 220–221° (ethanol), $[\alpha]_D$ - 13.5° (c 1, DMSO).

(b) 4-(2-*acetamido*-3,4,6- *tri*-O-*acetyl*-2- *deoxy*-β-D-*glucopyranosyloxy*) *benzaldehyde* was obtained similarly in 47% yield, $[\alpha]_D$ -19° (*c* 1, $CHCl_3$).

(c) 4-(2-*acetamido*-3,4,6- *tri*-O-*acetyl*-2-*deoxy*-β-D- glyco-*pyranosyloxy*)-3,5- *dimethoxybenzaldehyde* was obtained similarly in 15% yield m.p. 239 - 240° (methanol), $[\alpha]_D$ - 3.8° (*c*, 2 DMSO)

(d) 4-(2,3,4,6-*tetra*- O-*acetyl*-β-D *glucopyranosyloxy*)-3- *methoxybenzaldehyde* was obtained from 2,3,4,6-tetra-O-acetyl- α-D-glucopyranosyl bromide in 62% yield, m.p. 135 – 136° (ethanol), $[\alpha]_D$ -24.2° (*c* 1.1, $CHCl_3$).

(e) 4-0(2,3,4,6-*tetra*-O- *acetyl*-ß-D- *galactopyranosyloxy*) -3-*methoxybenzaldehyde* was obtained from 2,3,4,6- *tetra*-O-acetyl-α-D- galactopyranosyl bromide initially as a syrup (47%) which crystallised and was recrystallised with difficulty from ethanol-ether-light petroleum, m.p. 148–149°, $[\alpha]_D$ - 4.4° (*c* 1.1, $CHCl_3$).

(f) 4-(2,3,4,6-*tetra*-O- *acetyl*-α-D- *mannopyranosyloxy*) -3- *methoxybenzaldehyde* was obtained as a syrup in 14% yield from 2,3,4,6-tetra-*O*- acetyl-α-D- mannopyranosyl bromide (15), $[\alpha]_D$ + 15.9° (*c* 1.4, $CHCl_3$).

II O-Deacetylation of hydroxy-benzaldehyde glycosides

In the next stage of the preparation of the nitrostyryl enzyme substrates certain of the hydroxy-benzaldehyde glycosides are O-deactylated by treatment with methanolic sodium methoxide. Solutions of the acetylated glycosides (15 m mole) in methanol (50 – 500 ml) were treated with *M*-methanolic sodium methoxide (0.5 – 2 ml) and the solutions allowed to stand at room temperature for 20 – 30 minutes, or until reaction had been completed as indicated by t.l.c. determination. The solutions were then passed through pads of silica gel to remove sodium ions, and then evaporated to dryness.

(1) *4-Acetoxy-3-methoxy- ω-nitroxtyrene*

Vannillyl acetate (19.4g, 100 mmole) was reacted with nitromethane, ammonium acetate and acetic acid, as in method A part III of Example 1, for a period of 2–3 days. The red coloured reaction mixture was then diluted with water and the resultant oil layer extracted with chloroform. The chloroform extract was dried ($MgSO_4$) and evaporated to dryness, and the resulting solid recrystallised from chloroform-ethanol to give the acetate, m.p. 158–162.2°, (20g, 17%).

Alternatively the 3-methoxy-ω- nitrostyryl acetate was prepared from the corresponding hydroxy-nitrostyrene. 4-hydroxy-3-methoxy- ω-nitrostyrene (15g, 0.77 mmole) was added to acetic anhydride (15 ml) and the mixture heated under reflux for 1½h. The mixture was then poured into ice-water and the precipitated solid filtered off and recrystallised from chloroform-ethanol to give the acetate (1.7g, 93%), m.p. 163-165° identical to that prepared above.

The above ω nitrostyryl acetate is suitable for use in reagents and the method of the invention for detection and determination of acetate esterases, and on interaction with such enzymes gives rise to the release of 4-hydroxy-3- methoxy-ω- nitrostyrene which exhibits a strong red colouration under alkaline conditions.

(2) *4-Propyloxy-3-methoxy- ω-nitrostyrene*

4-hydroxy-3-methoxy- ω-nitrostyrene (3.9g, 20 mmole) was dissolved in dry pyridine (15 ml) and propionyl chloride (2.8g, *c* 30 mmole) added. The reaction mixture became warm and a dark red precipitate was formed. After *ca* 5 min. the mixture was poured into ice-water and the resulting yellow precipitate filtered off, washed well with ethanol and recrystallised from chloroform-ethanol to give the propionate (4.8g, 95%), m.p. 101–104.5°. The propionate is suitable for use in the detection of propionate esterases and similarly give rise to the formation of the 4-hydroxy-3-methoxy-ω-nitrostyrene on interaction with the enzyme.

(3) *4-(β-nitroethenyl)- 3-methoxyphenyl palmitate*

4-hydroxy-3-methoxy-ω-nitrostyrene (5.2g, 25 mmole) was dissolved in pyridine (20 ml) and palmitoyl chloride (8g, 29 mmole) added. A yellow solid was formed immediately and the reaction mixture was then heated at reflux for *c* 5 min; the reaction mixture was poured into ice-water and the resulting yellow solid filtered off. The product was stirred with dilute (10^{-4} molar) sodium hydroxide to remove free phenol, filtered off, washed well with water and ethanol, and then recrystallised from chloroform-ethanol, m.p. 96–99° (9.1 g, 84%). This product is suitable for use in the detection and determination of palmitate esterases, releasing 4-hydroxy-3- methoxy-nitrostyrene on interaction with the enzyme.

(4) *4-(β-nitroethenyl)- phenyl palmitate*

Palmitoyl chloride (3.6g) was added to dry pyridine (20 ml); there was an immediate precipitation. To this mixture was added 4-hydroxy-ω- nitrostyrene (1.65 g, 10 mmole) and the mixture heated at reflux for 1–2 min. The mixture was then poured into ice-water and the yellow amorphous precipitate filtered off. A solution of the precipitate in a mixture of chloroform and DMSO afforded seed crystals of the ester and the bulk was crystallised by dissolving in boiling methylene chloride and adding ethanol. When all the methylene chloride had evaporated, the ethanolic solution was seeded and gave the palmitate as pale fawn crystals, m.p. 83–84° (2 g, 50%). Similarly this product is suitable for detection and determination of palmitate esterases, though releases the 4-hydroxy-ω- nitrostyrene on interaction with the enzyme.

(5) *4-(β-nitroethenyl)- 3-methoxyphenyl phosphate (as monosodium salt mono hydrate)*

To vigorously stirred ice-cooled solution of phosphoryl chloride (6.2 g, 40 mmole) in pyridine (40 ml) was added dropwise over 45 min a solution of 4-hydroxy-3- methoxy-ω- nitrostyrene (7.8 g, 40 mmole) in pyridine (20 ml). The reaction mixture was then decomposed by the dropwise addition of aqueous pyridine (50 ml, containing 10% water) during which some solid separated. Dropwise addition of *2M*-sodium hydroxide (20 ml, 40 mmole) initially resulted in a clear solution, followed the separation of an amorphous mono sodium salt, as the hydrate (7.1 g, 59%), m.p.>250°. The salt had a low solubility in water, and is suitable for detection and determination of phosphatases.

(6) *4-(β-nitroethenyl)- 3-methoxyphenyl phosphate (as its disodium salt).*

The above reaction (5) is repeated except that the decomposed reaction mixture was poured into a concentrated solution of caustic soda (6g) in water. The product initially separated out as an oil which solidified to an amorphous solid (11g, 86%) m.p.>250°. It was more soluble in water than the monosodium salt and dissolved easily in boiling water, with a little decomposition, and reprecipitated on cooling to give a pale yellow solid. A 2m molar solution of the disodium salt could be made readily.

Example 3

A range of hydroxy-ω- nitrostyrenes was also prepared to determine and compare the colour properties of these compounds, being examples of compounds falling within the general formula X-A-Y-NO_2 i.e. compounds which may be formed as a result of interaction of the reagent of the invention with the corresponding enzyme.

The general preparative procedure used was as follows. The corresponding hydroxybenzaldehyde (40 mmole) was dissolved in ethanol (200 ml) and ammonium acetate (12 g), nitromethane (32 ml) and acetic acid was added. The reaction mixture was either stirred overnight at room temperature or heated under reflux for 15–60 minutes. In some cases the ω-nitrostyrene crystallised from the cooled reaction mixture, or crystallised when water was added to the cooled reaction mixture. In cases where no crystalline product could be isolated by these methods, the product was extracted with ether, which was washed well

the devices in a vertical orientation. The walls of the lower visualisation compartments 2 are of clear material, such as clear plastic, to permit observation of the DEAE cellulose and any coloured band absorbed thereon.

The upper incubation compartments 1 contain an appropriate quantity of the glycoside substance (not shown) either in a suitable solvent or absorbed into cellulose or other suitable absorbent e.g. 100 mg of microcrystalline cellulose (Avicell) containing 3% glycoside. The incubation compartments 1 are fitted with screw caps and have thick PVC or polystyrene walls 9 to insulate the urine during incubation, the walls 9 being marked with a line 10 to indicate the quantity of urine which is to be introduced for incubation.

In diagram, Figures 1 and 2, the upper and lower compartments 1 and 2 are shown without a connecting passageway between them.

With reference to Figure 1, the compartments 1 and 2 are held together at interfacial surfaces by pivot pin 11. The base of the incubation compartment 1 has an eccentrically located outlet 12 and the top of the visualisation compartment has an inlet 13 eccentrically located at the same radius from the pivot 11 as outlet 12. Mutual rotation of the upper 1 and lower 2 compartments brings the outlet 12 and inlet 13 into coincidence with one another providing a passageway connecting the compartments through which incubated urine can flow. In use, fresh urine is introduced into compartment 1 up to the level of the mark 10, outlet 12 and inlet 13 being out of coincidence with one another. The screw cap 8 is screwed in place and the urine is incubated for a period which depends upon the temperature though is usually about 15 to 20 minutes. The compartments are then mutually rotated bringing outlet 12 and inlet 13 into register with one another and incubated urine drains from the incubation compartment 1 to the visualisation compartment 2. 2-methoxy-4- (2-nitro-(E)-ethenyl) phenol released by the glycoside substrate as a result of NAG present in the urine gives rise to the formation of a bright scarlet red band about .5cm below the top of the DEAE cellulose column 3.

With reference to Figure 2, compartments 1 and 2 are held together in screw-threaded engagement by screw thread 16 around the bottom of compartment 1 and screw thread 17 around the inside of tubular extension 18 projecting from the top of compartment 2. A passageway between compartments 1 and 2 is provided by tubular extension 14 which projects from the bottom of compartment 1. Prior to use passageway 14 is closed by its abutment with the weakened section 15 in the top of compartment 2. In use, urine is introduced to compartment 1 and incubated as for the device of Figure 1. On completion of incubation, however, compartments 1 and 2 are screwed together and the tubular extension 14 breaks through the weakened section 15 permitting incubated urine to flow into compartment 2. Visualisation of the phenol released by the substrate is as for the device of Figure 1.

Both devices provided simple means by which "kidney transplant" patients may test themselves in their own homes, or general screening tests may be easily conducted, without access to sophisticated monitoring apparatus. Other enzymes besides NAG may be assayed using similar techniques and devices.

CLAIMS

1. An enzyme assay reagent comprising a substance which is capable of interaction with the enzyme to give rise to the formation of a compound of formula X-A-Y-NO_2, wherein A comprises an aromatic nucleus, X comprises an auxochromic group and Y comprises an unsaturated group which is capable of transmitting electron resonance between the aromatic nucleus and the nitro substituent, said compound *per se* being capable of producing a visual signal which is easily discernible by eye.

2. A reagent according to Claim 1, comprising a substance of formula S-X-A-Y-NO_2, wherein S comprises the enzyme substrate portion of the reagent.

3. A reagent according to Claim 1 or 2, comprising an appropriate carboxylate, phosphate, diphosphate, or sulphate ester for assay of corresponding carboxylate esterases and lipases, acid and alkaline phosphatases, phosphodiesterases or sulphatases.

4. A reagent according to Claim 1 or 2, comprising a glycoside for assay of the corresponding glycosidase.

5. A reagent according to Claim 4, comprising an α or β-glucopyranosyl, -glactoyranosyl, or -mannopyranosyl glycoside for assay of the corresponding glycosidase.

6. A reagent according to Claim 5, for assay of N-acetyl-β-D- glucosaminidase comprising the corresponding glucopyranosyl glycoside.

7. A reagent according to any of the preceding claims, in which the group A comprises a benzene nucleus.

8. A reagent according to Claim 7, comprising a substrate for the enzyme which, on interaction with the enzyme, gives rise to the formation of a compound of formula I

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